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Bley Folly, Brenda

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Chapter 1

The role of fructose-1,6-bisphosphate in metabolic regulation

Brenda Bley Folly, Nadia Huisjes and Matthias Heinemann.

Molecular Systems Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands.

Metabolism and its regulation

Metabolism describes the biochemical processes that provide energy and building blocks for growth and maintenance of any living being, and thus resides at the core of life. Therefore, competitiveness and survival depends on a fast adaptation of metabolic operations in response to environmental changes. In the classical view, extra- and intracellular nutrients are sensed by specific transmembrane or intracellular receptors and the information is transferred to the cellular regulatory machineries. However, a novel concept of metabolic regulation has been proposed recently[1,2]. In this type of regulation the availability of extracellular nutrients is sensed indirectly via metabolic fluxes. Changes in the kind or concentration of available nutrients lead to alterations in intracellular metabolic fluxes, which in turn are sensed by flux sensing systems and allow the regulation of the metabolism accordingly[3].

Intracellular metabolic fluxes are sensed via so called flux signalling metabolites, i.e. metabolites, whose levels correlate with the flux through metabolic pathways[1]. In order to translate the metabolic flux into an action, the flux signals have to be converted to cellular regulation mechanisms. For instance, flux signalling metabolites bind and modulate the activity of transcription factors, which in turn modulate gene expression in a flux-dependent manner[1].

One central metabolite indicated to act as a flux signalling metabolite is fructose-1,6-bisphosphate (FBP), an intermediate of glycolysis. Over a wide range of glycolytic fluxes, which can be obtained, for instance, by growing cells on different carbon sources, this metabolite was found to correlate with the flux through glycolysis[2]. FBP is known to control the activity of transcription factors[4,1], enzymes of glycolysis and gluconeogenesis[5,6], as well as enzymes from other cellular processes.

A metabolic flux-sensing system offers a robust type of metabolic regulation, since it allows cells to adapt their metabolism in response to a wide range of nutrients via a universal system and does not require the presence of nutrient-specific receptors. This concept was shown to be promising in a synthetic system[7], and has been described as a metabolic regulatory system in *Escherichia coli* and *Saccharomyces cerevisiae*[1–3,8].

FBP and its interactions

FBP correlates with glycolytic flux in a vast range of different organisms, such as *E. coli*, yeast and mammalian cells[2,3,7,8]. This suggests that the concept of flux-sensing and flux-dependent regulation might have formed early in evolution. In order to understand the role of FBP in the flux-dependent regulation of metabolism, in the following, we review the current knowledge about the regulatory network of FBP with effectors involved in various cellular processes across different organisms. Specifically, interactions of FBP with enzymes, proteins involved in signalling pathways and transcription factors will be described here.

Interactions of FBP with enzymes of the central carbon metabolism

As FBP is an intermediate of glycolysis, it is conceivable that FBP interacts with and influences the activity of enzymes of the central carbon metabolism. Therefore, we performed a comprehensive literature search to generate an overview of the interactions between FBP and these enzymes (Figure 1). The global trend shows that FBP activates glycolytic enzymes and deactivates gluconeogenic enzymes. This is in agreement with the fact that under high glycolytic flux, the intracellular concentration of FBP is increased, resulting in repression of gluconeogenenic enzymes. Next to this, it has been reported that glycogen breakdown is inhibited by FBP, while glycogen synthesis is stimulated.

Interactions of FBP with proteins of other metabolic processes

Besides enzymes of central carbon metabolism, FBP was found to modulate the activity of various enzymes involved in several other metabolic processes, such as amino acid synthesis, nucleotide synthesis and lipid metabolism (Table 1). In general, FBP has an activating influence on several enzymes involved in the lipid metabolism, but inhibits the activity of different enzymes responsible for nucleotide synthesis.

Interactions of FBP with proteins from the mitochondrial respiration

It seems that FBP also has a role in controlling mitochondrial respiration. In *S. cerevisiae*, an increase in glycolytic flux induces a metabolic switch from respiration to fermentation, under aerobic conditions. This effect has also been observed in fast growing tumour cells, in highly proliferating non-tumour cells[24], other *Crabtree* positive yeast strains, and some bacterial species[24–26]. In a recent study performed in *S. cerevisiae*, it was observed that FBP caused the inhibition of mitochondrial respiratory chain, resulting in a decrease in the respiration in these cells[27]. The same effect was also observed in isolated rat liver mitochondria[27]. Next to that, FBP was also found to close the mitochondrial unspecific

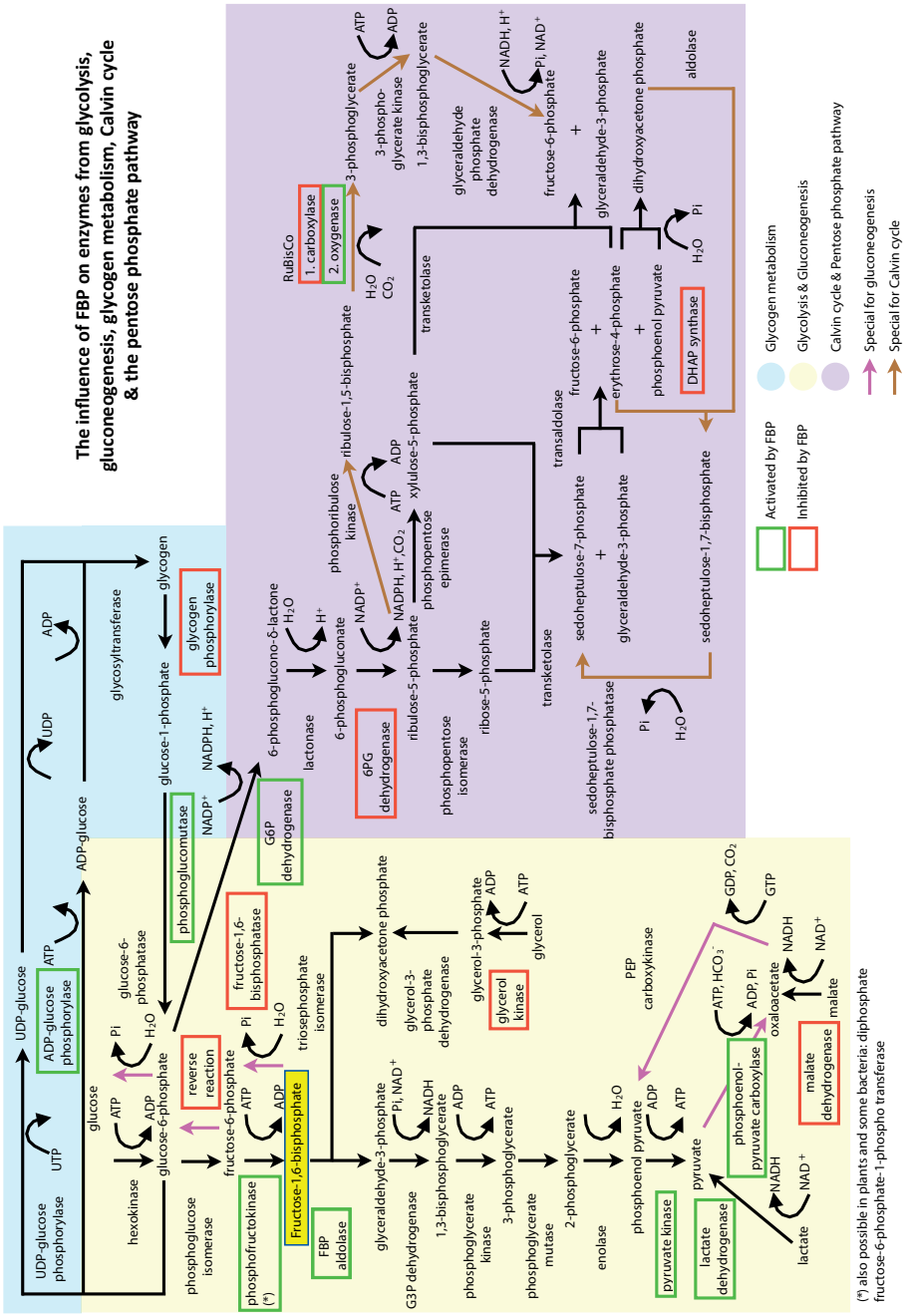

Figure 1. Overview of direct interactions of FBP with proteins from core carbon metabolism. In this figure the glycogen metabolism, glycolysis, gluconeogenesis, Calvin cycle and pentose phosphate pathway are shown. This figure was based on data from 12 organisms, including bacteria, yeast and mammals. References per enzyme can be found in the Supplementary Table 1. Pathway information is based on Berg *et al*[9].

channels (MUC), responsible for the regulation of the oxidative phosphorylation, and to inhibit the rate of oxygen consumption in *S. cerevisiae*[28]. The respiration inhibition caused by FBP is targeted on two complexes of the mitochondrial respiratory chain: complex III and complex IV[29]. In mammals, the mitochondrial permeability transition triggers cell death programs, while closed MUC results in resistance to apoptosis. In addition, closed MUC inhibits apoptosis and results in unregulated cell growth, which may indicate a causal relationship between the immortalization of tumour cells and the cytoplasmic accumulation of FBP, as observed in hepatoma cells, Ehrlich ascites and yeast *Crabtree* positive cells[27,28,30]. Thus, the respiration inhibition caused by FBP, is connected to its role in regulating the mitochondrial unspecific channels.

Interactions of FBP with transcription factors

FBP does not only regulate the activity of enzymes, but also of transcription factors (TFs), and thereby influences global gene expression. TFs bind to specific DNA operator sequences and activate or repress the expression of different genes. The activity of TFs can be regulated in different ways, for instance by posttranslational modification or interaction with ligands, which in turn lead to conformational changes that alter the affinity of the TFs for their target DNA operator[31].

FBP is known to regulate different transcription factors in various organisms. In *Bacillus subtilis* FBP is a well described regulator of the transcription factors 'central glycolytic gene repressor' (CggR) and 'catabolite control protein A' (CcpA)[32], members of the LacI-GalR family of transcription regulators and pleiotropic control proteins for catabolite regulation of genes[33]. Next to the interaction of FBP with CggR and CcpA, it was suggested that FBP might also be involved in the transcriptional control of lactate dehydrogenase (LDH) and pyruvate formate-lyase in *Streptococcus bovis*[34]. It is worth to mention that the regulation of LDH expression was also indicated to be regulated by the TF CcpA[34].

CggR regulates the expression of the *gapA* operon including several glycolytic genes. The interaction of CggR with the operon *gapA* represses transcription. However, the binding affinity of CggR to its target DNA is lowered upon binding of FBP, leading to enhanced trancription[35,36]. CggR contains two FBP binding sites with different affinities. Binding of FBP to the high affinity[37,38] binding site causes a conformational change in the CggR/DNA complex that stabilizes the CggR dimer, but does not influence the affin-

Table 1. Effect of the interactions of FBP with enzymes from different metabolic processes.

Enzyme	Cell process	Effect of FBP	Organism	Reference
Glutamine synthetase adenylyltransferase	Adenylation of glutamine synthetase	Inhibits	*E. coli*	[10]
Glutamine synthetase	Glutamine production	Activates***	Rat	[11]
Adenine phosphoribosyl transferase	Conversion of adenine to AMP (purine pathway)	Inhibits	Rat	[12]
Adenylosuccinate synthase	Conversion of inosine to AMP (purine pathway)	Inhibits	Rat	[13]
Adenylyl cyclase	cAMP production	Inhibits / Activates *	*Streptococcus*	[14]
Acetyl CoA carboxylase	Lipid metabolism	Activates	*Saccharomyces carlsbergensis*	[15]
Fatty acid synthetase	Lipid metabolism	Activates	Pigeon, human, *E. coli*	[16–19]
Phospholipid synthesis	Lipid metabolism	Activates	Rat	[19]
Lipolysis	Lipid metabolism	Activates	Rat	[20]
β-Galactosidase	Sugar break-down	Destabilizes	*E. coli*	[21]
Isocitrate lyase	Conversion of isocitrate (glyoxylate cycle)	Inhibits	*Pseudomonas indigofera*	[22]
HPr kinase	Phosphorylation of HPr or Crh**	Activates	*Bacillus subtilis*	[23]

* Depending on the concentration of FBP: inhibits at high concentration, activates at low concentration.

** HPr: histidine containing protein, Crh: catabolite repression HPr.

*** Under hypoxic conditions.

ity to the DNA operator site. Upon FBP binding to the lower affinity site, conformational changes within CggR reduce its affinity for the DNA operator[37].

CcpA modulates carbon catabolite regulation (CCR) either by repression or activation of over 300 target genes important for carbon metabolism in *Bacillus subtilis*, including enzymes, transporters and several transcription factors[39]. CcpA is quite unusual amongst the LacI-GalR proteins, since it uses phosphoproteins from the HPr family as cofactor (either HPr or Chr). Although these phosphoproteins play an essential role in the regulation of this transcription factor, it was described that CcpA activity is regulated by a combination of different signals, amongst others small molecules as FBP and glucose 6-phosphate (G6P). These metabolites function as corepressors to fine-tune the response of CcpA adjusted to the metabolic state of the cell. Studies have demonstrated that FBP and G6P stimulate specific binding of CcpA to its DNA operator[39].

The transcription factor 'catabolite repressor activator' (Cra) from *E. coli* has also been suggested to be regulated by FBP[40,41] as well as by fructose-1-phosphate (F1P)[40,41]. Cra, a member of the GalR-LacI superfamily of DNA-binding transcriptional regulators[42], controls the transcription of several operons in enteric bacteria concerning carbon and energy metabolism. Among others it is responsible for the switch between glycolysis and gluconeogenesis by activating transcription of genes encoding biosynthetic and oxidative enzymes involved in the Krebs cycle, glyoxylate shunt and gluconeogenesis, and repressing transcription of genes involved in glycolysis[40,41,43,44]. Upon binding of F1P or FBP, Cra is displaced from the DNA binding sites, leading to an increase in the expression of glycolytic genes, and repression of the gluconeogenetic genes, which implies acceleration of the glycolytic fluxes[4].

The role of FBP in the regulation of Cra, however, is still a point of debate. Some studies claim that Cra is regulated by both F1P and FBP[40,41], although these studies did not provide any experimental evidence to prove direct interaction between Cra and FBP[40]. DNA shift assay experiments with a labeled DNA fragment bearing a Cra operator sequence[40,43] indicated binding of F1P, but not FBP. Other studies suggested that the *in vitro* response of Cra to FBP is due to a possible contamination of FBP with F1P[40]. However, some authors provided evidence that FBP is indeed an authentic regulator of Cra[1,3,43]. Up to now, the consensus is that F1P is the major agonist of Cra, and that FBP can also modulate its activity, albeit at much higher concentrations.

Interactions of FBP with proteins from signalling pathways

FBP has been found to play a role in the regulation of proteins from signalling pathways, for instance the epidermal growth factor receptor (EGFR) in triple-negative breast cancer cells (TNBC)[45]. EGFR is one of the major regulators of cell proliferation, survival and metabolism, and was found to be overexpressed in some types of cancer cells[46]. EGF signalling in TNBC cells activates the first step of glycolysis and slows down the last step of glycolysis, causing accumulation of metabolic intermediates within this pathway. Furthermore, FBP was found to bind directly to EGFR and enhances its activity, thereby increasing lactate excretion in TNBC, which eventually leads to inhibition of local cytotoxic T- cell activity. Since cytotoxic T-cells usually kill cancer cells, this would boost tumour growth[45].

Recently, a new interaction between FBP and a signalling protein was identified. Ras proteins are small GTPases involved in cellular signal transduction. Once activated, Ras induces the activation of other proteins responsible for cell growth and differentiation. Mutated Ras genes, encoding overactive Ras proteins, are the most common oncogenes found in cancer cells[47]. Recently, it was found that FBP, in combination with guanine nucleotide, activates Ras in *S. cerevisiae*[48]. The authors describe a conserved mechanism from yeast to mammals, where FBP binds the proteins Cdc25/Sos1, essential for the activation of Ras. This mechanism couples increased glycolytic flux to increased Ras proto-oncoprotein activity, and suggests that FBP can be involved in the regulation of cell proliferation in cancer cells[48].

Perturbations of intracellular FBP levels

The previous sections clearly indicates the importance of FBP as a flux-signalling metabolite. Therefore, the intracellular concentration of FBP plays an important role in regulatory mechanisms, which eventually could influence the fate of a cell. This section provides an overview of phenotypic effects caused by altered FBP levels, reflecting altered glycolytic fluxes. Such information will provide us with a better understanding of FBP as a regulator of metabolism. Next to altering glycolytic fluxes (for instance, by use of different nutrients), different possibilities exist to manipulate cellular FBP levels: intracellular FBP levels can be perturbed by altering the expression or the activity of enzymes responsible for the production or the conversion of FBP into other metabolites, or by the administration of FBP.

Effects of increased FBP levels

Different studies involving the administration of FBP have been performed (Table 2). One of these studies used mammalian cell extracts to determine the effect of phosphorylated sugars in protein synthesis[49]. Cell extracts that are not supplemented with sugars show a decline in protein synthesis and a decrease in the amount of Met-tRNAf that binds to 40S ribosomal subunits. The addition of the phosphorylated sugars to these cell extracts, especially the addition of FBP, increased the binding activity of Met-$tRNA_f$ and allowed protein synthesis to resume. Another study analysed the intraperitoneal administration of galactosamine, known to cause reversible liver cell injuries, in combination with FBP, F1P or F6P[50]. The cell damages, monitored through changes in the serum enzymatic activities, were prevented only when galactosamine was co-administrated with FBP. Another study analyzed the effects of infusion of FBP via brachial vein in patients[51]. Here, they observed a slight decrease in heart and respiratory rate, an increase in inorganic phosphate and intra erythrocytic ATP concentration, as well as a decrease in plasma cholesterol and triglycerides. In this study the authors suggested that FBP was responsible for enhancing glycolysis.

Table 2. Compilation of studies determining the effects of administration of FBP in different cell types.

Cell type	Effects observed / Metabolic behaviour	Organism	Reference
Administration of FBP			
Ascites, HeLa, myeloma and reticulocytes	• FBP stimulate protein synthesis in extracts from these types of mammalian cells.	Mammalian	49
Intraperitoneal administration of galactosamine and FBP in liver cells	• This simultaneous administration prevented liver cell death.	Rat	50
Infusion of FBP via brachial vein	• Slight decrease in heart and respiratory rate. • Increase in inorganic phosphate and intra erythrocytic concentration of ATP. • Decrease in plasma cholesterol and triglycerides.	Humans	51

Table 3 summarizes the effects of knockout studies that result in increased intracellular levels of FBP. The metabolic response due to the knockout of FBPase, the enzyme responsible for converting FBP in fructose 6-phosphate in gluconeogenesis, was studied

in *A. thaliana*, where two isoforms of the enzyme are present: one located in the cytosol and one inside the chloroplasts[52]. Knockout strains of each isoform and the corresponding double mutants had an overall accumulation of FBP, the highest in the double deletion mutant. The absence of the cytosolic form resulted in accumulation of hexose phosphates and increased levels of starch. The knockout of the chloroplastic isoform had a profound effect on photosynthetic carbon metabolism and photorespiration, showed decreased levels of intracellular sugars, presented cell structural deficiencies and reduced plant growth. The double mutant showed a decline in sucrose content.

Table 3. Compilation of knockout studies determining the effects of high levels of FBP in different cell processes.

Enzyme	Effects observed / Metabolic behaviour	Organism	Reference
Knockout			
FBPase cytosolic	• Accumulation of FBP and hexose phosphate. • Increase in the starch levels.	*Arabidopsis thaliana*	52
FBPase chloropastic	• Accumulation of FBP, triose-phosphates, and 3-phosphoglyceric acid. • Decline in the levels of hexose-phosphates and sugars, including sucrose, glucose, fructose, and trehalose (signalling) and maltose (starch degradation). • Induced cell structural deficiencies, and reduced plant growth.	*Arabidopsis thaliana*	52
FBPase cytosolic and chloroplastic double mutant	• Higher increase in FBP accumulation. • Decline in sucrose content.	*Arabidopsis thaliana*	52

Effects of decreased FBP levels

Intracellular levels of FBP can be decreased by enhancing the production of the enzymes aldolase or FBPase, by deletion of phosphofructokinase, or yet as a response to the processing of certain carbon sources. In Table 4 the effects of decreased intracellular levels of FBP, observed in distinct studies, are summarized.

The overexpression of fructose-1,6-bisphosphate aldolase was studied in *Streptococcus bovis*[34]. The intracellular concentration of FBP considerably decreased as compared to the parental strain, whereas DHAP and GAP were slightly higher and the ratio of formate to lactate production was increased. The authors suggested that the synthesis

of lactate dehydrogenase might be indirectly regulated by FBP, since the transcription of the *ldh* gene in *S. bovis* is presumed to be regulated by CcpA, a transcription factor known to be regulated by FBP in *B. subtilis*[34].

The overexpression of FBPase was studied in *C. glutamicum*[53,54], where a decrease in FBP concentration was observed in cells growing on sucrose. In this study a significant improvement in lysine production was observed, suggested to occur due to the redirection of carbon from glycolysis toward the PPP and an increased NADPH supply. Additionally these cells showed an increased growth rate, substrate uptake rate and biomass yield.

The overexpression of FBPase was also studied in pancreatic β cells, using mouse cells overexpressing human FBPase[55]. Lower levels of FBP and high levels of F6P were detected in cells growing in the presence of high glucose concentrations. A decrease in glucose utilization and energy production resulted in reduced insulin secretion of these cells.

The effects of overexpressing FBPase were also analysed in hepatocellular carcinoma[56]. These cells are known to have lower expression of FBPase, which is associated with advanced tumour stage and higher tumour recurrence rates. Upon increased levels of FBPase, a decrease in the concentration of FBP and G6P was observed, and the tumour growth and the glucose uptake rate were inhibited.

The recent study from Zhang and co-authors, explored the perturbations in the metabolism as a response to different carbon sources[57]. In this study, performed in mouse embryo fibroblasts grown in low-glucose medium, decreased intracellular levels of FBP were found – consistent with the possibly decreased glycolytic flux. Under these conditions, it was also observed that the enzyme AMP-activated protein kinase (AMPK) was activated. In order to test whether FBP was responsible for repressing the activation of AMPK, FBP was introduced into glucose-starved cells permeabilized with streptolysin O, and a decrease in AMPK activation was observed.

Taken together, this overview shows that FBP regulates the activity of various proteins involved in a wide range of metabolic processes. Moreover, alterations in the intracellular levels of FBP caused by perturbations, trigger metabolic alterations accordingly. Together with the fact that FBP levels correlate with the metabolic flux through glycolysis,

the information gathered provide strong evidences that FBP is part of a metabolic flux sensing systems, where it acts as a signalling metabolite, regulating metabolism in a flux-dependent manner.

Table 4. Compilation of studies determining the effects of low levels of FBP in different cell processes.

Enzyme / Cell type	Effects observed / Metabolic behaviour	Organism/ cells	Reference
Overexpression			
Aldolase	• Lower FBP levels in cells growing on glucose. • DHAP and GAP levels were higher. • ATP and ADP levels were lower.	*Streptococcus bovis*	[34]
FBPase	• Increase in FBP concentration in cell growing on sucrose. • Increase in lysine production on sucrose.	*C. glutamicum*	[53,54]
FBPase (mouse cells overexpressing human FBPase)	• Lower FBP and ATP concentration. • Lower glucose utilization and energy production. • Result in insulin secretion.	Human pancreatic β cells	[55]
FBPase	• Lower FBP and G6P concentration. • Reduced glucose uptake rate, reduced glycolysis. • Tumour growth was inhibited.	Human hepatocellular carcinoma cells	[56]
Knockdown			
Aldolases	• AMPK activation even in glucose (i.e. high FBP levels). • FBP represses the activation of AMPK only via interaction with aldolase.	Mouse embryo fibroblast	[57]

Methods to study protein-metabolite interactions

The interaction between metabolites and proteins is crucial for the regulation of distinct cellular processes. Identifying protein-(flux-signalling) metabolite interactions would provide essential information for understanding the downstream targets of flux signals. However, the vast chemical diversity of metabolites, combined with the fact that protein-metabolite interactions are often weak and transient, makes it extremely challenging to identify these interactions in a high throughput manner. Due to the lack of generally applicable systematic approaches, protein-metabolite interactions are

often still identified using laborious *in vitro* activity assays, such as isothermal titration calorimetry, surface plasmon resonance and microscale thermophoresis. Recently, new methods that focus on the identification of protein-metabolite interactions in large-scale have been developed, and some of them are described below.

Methods such as thermal shift assays were originally designed to optimize recombinant protein stability, and are currently also used in high throughput screenings to identify new protein-metabolite interactions[58]. This technique detects differences in the stability of proteins in the presence and absence of certain metabolites, by relying on the fact that protein structures are more stable when bound to their corresponding ligand[59]. Thermal shift assay, also known as differential scanning fluorimetry[60,61] measures the increase in the fluorescence of the SYPRO orange dye, which interacts with hydrophobic parts of the proteins that are exposed upon temperature-induced denaturation. An interaction between the ligand and the protein of interest could increase thermal stability of the protein and thereby cause a delayed increase in fluorescence. Similar strategies, such as drug affinity responsive target stability (DARTS)[62] and a proteomics method called stability of proteins by rates of oxidation (SPROX)[63,64] have also being successfully used in the discovery of new interactions.

Another new strategy for the high throughout identification of interaction between metabolites and proteins is differential radial capillary action of ligand assay (DRaCALA), which requires radiolabeled metabolites.[65] This method makes use of the fact that proteins bind to nitrocellulose membranes and sequester radiolabeled metabolites, which are bound to the protein. One advantage of DRaCALA is that the raw cellular extracts containing an overexpressed protein of interest can be used without any further time-consuming protein purification. However, the costs and availability of radiolabelled metabolites can limit the use of this approach.

A recent method developed by Veyel and co-workers[66] was based on the supposition that metabolites that interact with proteins form stable complexes that will fractionate together when a size separation method is applied. In this study, *Arabidopsis thaliana* cells were lysed and the soluble fraction, containing several proteins and metabolites, was separated by size using analytical ultracentrifugation or size filtration columns. Metabolites that were not bound to proteins were separated in the low molecular weight fraction. With this methodology, the authors were able to detect several metabolites, indicating that the metabolites indeed formed stable, non-covalent complexes

with proteins, and can be recovered from protein-metabolite complexes. However, one limitation of this method is that the fractionation/separation of size is not sufficient to allow the specific identification between one protein and metabolite, since several proteins and several metabolites are analysed at the same time.

A recent proof-of-concept study used ligand-detected nuclear magnetic resonance (NMR) spectroscopy to systematically identify protein-metabolite interactions[67]. A key advantage of this method is the possibility to test the impact of several metabolites simultaneously on one purified protein. Different NMR methods that detect signals from low molecular weight ligands and changes in their properties upon binding to a protein were tested. From the three NMR methods tested, two methods, water-ligand observation with gradient spectroscopy (WaterLOGSY) and diffusion and relaxation-edited NMR (T1hro relaxation), showed high sensitivity and robustness. These experiments identified most of the already known interactions, and also revealed new interaction partners. However, when increasing amounts of different metabolites were used in the analysis, a decrease in the detection of interactions was observed. A possible explanation for that is that compounds may compete for the same binding site in the studied protein, increasing the likelihood of false negatives.

A large-scale study developed by Li and colleagues[68] used affinity protein purification and mass spectrometry (MS) assay for the identification of protein-metabolite interactions in yeast. They focused on interactions of hydrophobic metabolites with kinases and proteins from the ergosterol pathway. Previously known interactions were identified, together with several new ones. Many enzymes from the ergosterol pathway were found to interact with sterol intermediates, suggesting a regulatory role of these molecules in this pathway.

The use of FBP on a novel mass spectrometry based method for the identification of protein-metabolite interactions

Feng and co-workers[69] developed a method to identify condition-dependent *in vivo* protein-metabolite interactions by exploring the structural transitions of proteins in response to interaction with metabolites. In this method, proteins are first submitted to a digestion that cleaves off peptides present on the surface of the protein on a certain condition. Under a different condition, for instance, upon binding of a ligand, the conformation of the protein changes, and the same digestion results in the cleavage of different peptides.

In this study, they focused on yeast metabolism, and investigated interactions that occur when cells grow on glucose or ethanol. The proteome of these cells were extracted under non-denaturing conditions, and submitted to double digestion. First, the samples were digested by limited proteolysis (LiP), which generates peptides according to the structural conformation of the proteins in the specific condition. The second digestion of the proteome was performed by full trypsinization. A control sample, subjected only to trypsinization, was also prepared. The peptides generated were submitted for analysis using selected reaction monitoring (SRM)-MS. To identify structural alterations in the proteins due to different growth conditions, i.e. glucose or ethanol, the proteolytic patterns of the samples are compared after normalization with the control to correct for protein abundance between samples, incomplete trypsin specificity and endogenous protease cleavage.

To validate the method, the authors used the protein pyruvate kinase (Cdc19), which is known to be activated by FBP, whose concentration is 100-fold higher in glucose than in ethanol. The analysis of SRM showed that the peptides with largest fold changes between the glucose and the ethanol condition were located in the active site and the FBP binding site. To determine whether the alterations observed were indeed due to changes in the concentration of FBP, this metabolite was added to lysates from ethanol growing culture. The proteolytic pattern of Cdc19 was reverted to the one observed when in presence of glucose, and the activity of the enzyme was recovered. These findings validated the method and confirmed that FBP was the responsible for the structural alterations observed between these two conditions. It was also observed that the addition of FBP to lysates from ethanol growing cultures altered the proteolytic pattern of about 70 other proteins, including proteins previously described to be regulated by FBP, as well as proteins that were not known to bind FBP, suggesting that this metabolite interacts and modulates the activity of these proteins.

Aim and outline of this thesis

The aim of this thesis was to investigate the role of FBP as a flux-signaling metabolite and its participation in the regulation of the metabolism.

The MS-based method described above[69], resulted in a list of suggested interactions between FBP and proteins whose structures suffered alterations when in presence of FBP. In Chapter 2 and 3 some of these interactions were studied. In Chapter 2, we used

a biochemical approach to validate the interaction suggested between Hxk2 and FBP. Hxk2 was previously found to suffer conformational changes when in presence of FBP[69]. However, we did not find any indication of a direct interaction with FBP, therefore, we hypothesized that the structural alterations of Hxk2 could occur due to an interaction of FBP, by acting as a chelator, with metal ions that are important for the enzyme activity. In Chapter 3, seven other proteins that also showed structural changes in presence of FBP were biochemically tested to determine whether these changes were due to an interaction with FBP. Also for these proteins, our results indicate that there is no direct interaction of FBP with the studied proteins. In Chapter 4, we explored the role of FBP as a regulator of the transcription factors Cra and CggR. For CggR, we determined that millimolar concentrations of FBP are required to regulate the interaction of CggR with its DNA operator sequence. For Cra, we could finally establish that FBP does not interact with Cra, and do not regulate the activity of this transcription factor. In Chapter 5, we provide an outlook on the findings of this thesis, suggesting future research lines on the metabolism regulation.

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Supplementary information

Supplementary Table 1: Interactions of FBP with enzymes from glycolysis, gluconeogenesis, glycogen metabolism, Calvin cycle and pentose phosphate pathway.

Enzyme	Effect of FBP	Organism	Reference
Phosphofructokinase	Activates	Mammal, yeast, clostridia, lactobacilli	[5,6] [70]
Fructose-1,6-bisphosphate aldolase	Activates	*Pseudomonas aeruginosa*	[71,72]
Pyruvate kinase	Activates	Yeast, *E. coli*, mammal	[70,73,74]
Phosphoglucose isomerase	Inhibits reverse reaction	Rabbit	[75]
Fructose-1,6-bisphosphatase	Inhibits	Rabbit	[76]
Lactate dehydrogenase	Activates (prevents inactivation) Essential dependency	Streptococci, lactobacilli Thermal bacteria	[77,78] [79,80]
Glycerol kinase	Inhibits	*E. coli*	[81,81]
Diphosphate-fructose-6-phosphate-1-phosphotransferase	Activates	Mung beans, barley	[82,83]
Malate dehydrogenase	Inhibits	*Streptococcus faecalis*	[70,84]
Phosphoenol pyruvate carboxylase	Activates	*E. coli*	[85]
Glycogen phosphorylase	Inhibits	Mouse	[86]
Phosphoglucomutase	Activates	Rabbit	[87]
ADP-glucose pyrophosphorylase	Activates	*E. coli*, algae, plants	[88–90]
6-Phosphogluconate dehydrogenase	Inhibits	*Streptococcus faecalis*, Neurospora, yeast, rat, sheep	[91]
Glucose-6-phosphate dehydrogenase	Activates	Loach, yeast	[92]
Ribulose-1,5-bisphosphate carboxylase	Inhibits	Spinach, algae, corn	[93]
Ribulose-1,5-bisphosphate oxygenase	Activates	Spinach	[94]

Supplementary Table 1: Interactions of FBP with enzymes from glycolysis, gluconeogenesis, glycogen metabolism, Calvin cycle and pentose phosphate pathway. (continued)

Enzyme	Effect of FBP	Organism	Reference
2-Keto-3-deoxy-D-araboheptonic acid 7-phosphate (DHAP) synthase	Inhibits	*E. coli*	[95]